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Availability of extractives from various Norway spruce (*Picea abies*) stumps assortments

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ABSTRACT

Stumps and knotwood of Norway spruce (*Picea abies*) are valuable sources of wood extractives. Although lignans from knotwood have already been utilized in value-added products, the behavior and valorization of stump-derived extractives are less studied. In this study, the composition of lipophilic and hydrophilic extractives, particularly lignans, from various spruce stump samples (stump bottom, stump heart, and crushed stump samples) stored outside were studied. Lipophilic and hydrophilic extracts were separated with an accelerated solvent extraction (ASE) apparatus using *n*-hexane and hot water, respectively. The detailed extractives content of samples was then determined by gas chromatography equipped with a flame ionization detector and a mass detector (GC-FID/MS) and high-performance liquid chromatography (HPLC). In stump bottom samples, an apparent decrease in total dissolved solids was observed in all the major extractives groups during storage: lignans, sugars, stilbene–glucosides, organic acids, resin acids, fatty acids, diterpenoids, and sterols. While a definitive decrease in extractives could not be demonstrated due to the moderately high variation of extractives among different samples, a good indication of the accessibility of important extractives in weathered stumps was obtained. Of the identified hydrophilic extractives, 79% were lignans, 53% of them being composed of 7-hydroxymatairesinol (HMR), 16% conidendric acid, and 12% todolactol. After 12 weeks of storage, the total amount of lignans was 15.3 mg/g of dry matter in stump bottom, 17.0 mg/g of dry matter in stump heart samples, and 10.2 mg/g of dry matter in crushed stump samples.

KEYWORDS

Lignan; 7-hydroxymatairesinol; high-performance liquid chromatography; gas chromatography-mass spectrometry; stump extractives; Norway spruce

Introduction

In recent years, biomaterials containing high contents of extractives, such as forest industry side-stream wood bark, are commonly used for producing energy, but their utilization for other purposes is also gradually increasing. Traditionally, the extractives from aged and pine trees (especially stumps) comprise an interesting substance group, which has been utilized, for example, to prepare tar in the Nordic countries.[1] This manufacturing process was started in the forest three to four years before the actual production in the tar pit by removing bark from pine trees, thus inducing excessive oleoresin formation of the trees.[2] In the case of pine stumps, the stump hearts may become increasingly resin-hardened and thus rot-resistant after felling.[3] This has historically made them especially favorable materials for tar production. However, this kind of resin saturation over time does not seem to occur readily in spruce stumps. Hence, the critical question is if there is a good rationale for the recovery and further utilization of spruce stump extractives, and is it worth the cost.

The knotwood of many wood species has a high concentration of valuable extractives. For example, the knot wood of aspen has been found to contain increased amounts of flavonoids,[4] and the knotwood of pine lumber has been found to contain ten times higher terpenoid concentration compared to sapwood.[5] Similarly, the knotwood of Norway spruce (*Picea abies)* is known to be saturated by the fraction of lignans, the most prominent compound being 7-hydroxymatairesinol (HMR).[6] However, *P. abies* stumps are also known to contain high levels of lignans and stilbene–glucosides.[7] Lignans are natural

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polyphenolic antioxidants that have been attributed with various health benefits, such as anti-breast cancer and prostate cancer activity.[8,9] The prospect of utilizing leftover Norway spruce stumps as feedstock materials, similarly to how spruce knotwood is already being used, for producing value-added chemicals, offers an attractive possibility.

However, the development of spruce stump extractives-based products requires a broad chemical understanding of extractable compounds, together with the influence of stump aging on the content of extractives, which are of primary importance. The degradation of spruce bark, forest residues, and stumps was studied in the EU-funded research project called BioHub. This project's general aim was to understand better the effects of storage on the chemical composition of various forest industrial sidestreams and, consequently, to find ways to improve their procurement practices.

The main goal of this study was to increase the general knowledge and provide a broad picture of extractives behavior during stump storage by investigating the effects of outside storage on the chemical composition of various stump assortments. Our primary focus was on the most prominent compound fraction of stumps, namely, lignans.

Materials and methods

Storage studies and sampling

A mature, Norway spruce-dominated stand was clear-cut in Kannus, Finland, in early May 2017. The tree stumps were extracted and split into 2–3 segments with a standard stump rake attached to an excavator. This was followed by an immediate forwarding to a landing for storage during May 15–19, 2017. Stump segments were placed in $3 \times 10 \times 2.5$ m ($W \times L \times H$) piles. Care was used not to contaminate stump sections with the underlying soil by placing them on an older set of stumps. Three types of samples were collected: (a) crushed stump, (b) stump heart, and (c) stump bottom (Figure 1). Collection times were at the initiation of the study (zero-sample, taken on May 23–24, 2017), 4, 12, and 25 weeks after the initiation. The bottom and heart samples were collected using a chain saw without bar oil at each sampling time. These samples were debarked and comminuted to a smaller particle size with a 5.6 kW Murray Mulch Maker. The crushed stump samples were stored as whole until sampling and then crushed with a 708 kW Vermeer HG6800TX horizontal grinder. After comminution, all samples were closed in plastic bags and a cooler box for immediate transport to the laboratory cold storage at −20 °C for chemical analysis.

Sample pretreatment and basic characterization

The moisture content of fresh stump samples was determined by a standard method (CEN/TS 14774-2:2004).[10] The stump samples were dried at a temperature of 105 °C until a constant mass was achieved. All the measurements were performed in duplicate.

The stumps were lyophilized (for three days) and ground with a Retsch SM 100 cutting laboratory mill (Retsch GmbH, Haan, Germany) equipped with a bottom sieve with trapezoidal holes (perforation size <1.0 mm) for chemical analysis. Samples were stored in a frozen state (<−20 °C). The dry matter content of each lyophilized stump sample was determined by drying about 1 g of stump powder in a tared crucible in an oven at 105 °C oven overnight.

Chemicals

The solvents used in the sample preparation of extractives were analytical grade acetone (BDH), *n*-butanol (Merck), high-performance liquid chromatography (HPLC)-grade *n*-hexane (VWR), methyl *tert*-butyl ether (MTBE, Lab-Scan), pyridine (BDH), and 95% ethanol (EtOH, >94%, ETAX A, Altia Corporation). The silylation reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), were from Regis Technologies. HPLC-grade methanol (MeOH, Merck), HPLC-grade acetonitrile (VWR International), formic acid (≥98%; Sigma-Aldrich, Espoo, Finland), and *trans*-polydatin (99%, PhytoLab) were used in the HPLC analysis stump samples.

The compounds used as internal standards in the gas chromatography (GC) analysis of extractives were heneicosanoic acid (99%, Sigma), betulinol (≥98%, Sigma), cholesteryl margarate (≥97%, TCI America), and 1,3-dipalmitoyl-2-oleylglycerol (≥99%, Sigma). Other chemicals used in the analyses were NaOH (>98%, VWR), HCl (37%, VWR), Na_2CO_3 (≥99.8%, Sigma), H_2SO_4 (95–97%, Sigma), and bromocresol green (>95%, Sigma).

Separation of stump extractives

The extractions of stump samples were made via a Dionex Accelerated Solvent Extractor (ASE 100) using *n*-hexane and water as solvents to extract lipophilic and hydrophilic extractives, respectively. The extraction temperature was 120 °C, static extraction time

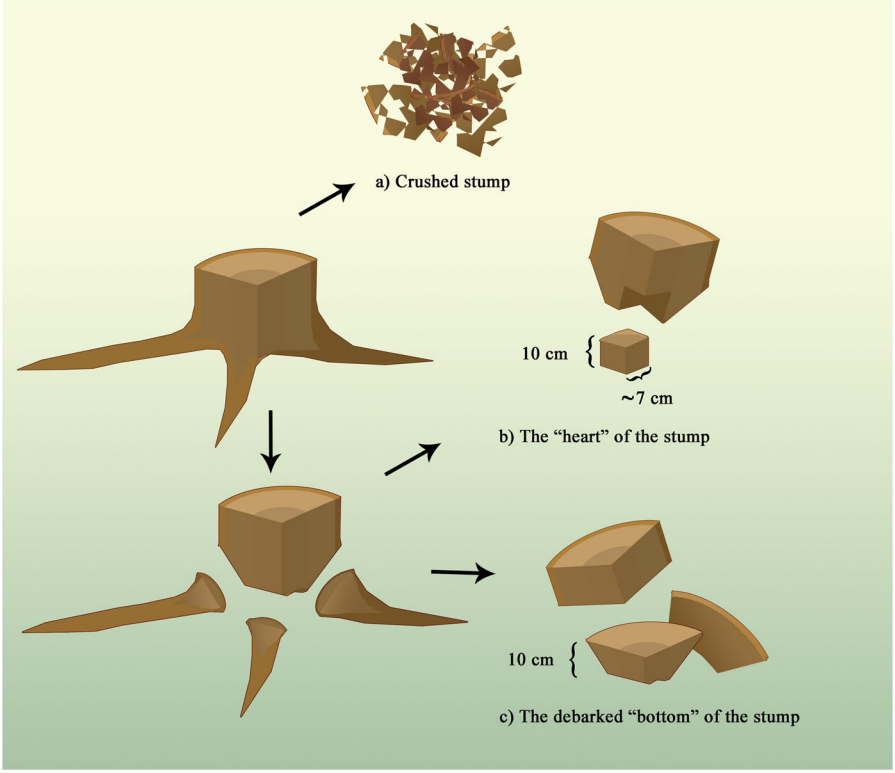

Figure 1. Preparation of the different stump samples for chemical analysis. (a) Crushed stump samples were prepared directly from pre-split stumps with roots intact. (b) The stump heart samples were cut as 10 cm × 7 cm × 7 cm pieces from the de-rooted stump, while (c) the stump bottom samples were cut at ~10 cm height from the bottom of the de-rooted stump and debarked.

was 10 min, extraction cell flush was 60%, nitrogen purge was 70 s, and extraction pressure was 1500 psi. Approximately, 2 g of dried stump powder was loaded to a 34-mL extraction cell plugged with a cellulose filter for each extraction. Each sample was first extracted with *n*-hexane and then with water. The extractions were performed in duplicates for each sample.

Gravimetric analysis of total dissolved solids and stock solutions

The total dissolved solids (TDS) of stump extracts were determined gravimetrically. The *n*-hexane extracts were evaporated to near dryness in a rotary evaporator, transferred to tared Kimax test tubes in acetone, and finally evaporated to dryness under nitrogen flow. The mass of the dried extract was the TDS of *n*-hexane extracts. Stock solution (100 mL) of the lipophilic extract was then prepared by dissolving the dried extract in acetone.

Stock solutions of the hydrophilic extract were prepared by diluting the raw extract to 100 mL with ultra-high quality (UHQ) water. Then, 10 mL of the stock solutions was lyophilized and the TDS of the hydrophilic extracts was determined based on the lyophilized sample mass.

Chromatographic analysis methods

Qualitative analysis by GC-MS

For qualitative analysis, 3 mg of extracts (based on dry mass) were dried (either by nitrogen flow or lyophilization) and dissolved in 500 μL of pyridine and 300 μL of the silylation reagent (BSTFA/TMCS (95/5, vol/vol)). The silylation was accelerated by keeping the sample in an oven at 70 °C for 1 h. The sample was then analyzed by GC-MS using an HP-5 column (30 m × 0.32 mm, with 0.25 μm film), injecting the sample at 290 °C and detecting the compounds with a mass selective detector (EI) at 300 °C. The temperature program was: at 100 °C (1.5 min), to 180 °C (6 °C/min) to 290 °C (4 °C/min), at 290 °C (13 min), to 300 °C (4 °C/min), and at 300 °C (20 min).

Quantitative analysis by GC-FID

For quantitative analysis of individual lignans, approximately 3 mg of stump extracts were dried together with 100 μg of internal standards (heneicosanoic acid and betulinol), the mixtures were dissolved in 500 μL of pyridine and 300 μL of the silylation reagent, and kept in an oven at 70 °C for 1 h. Long column GC-FID equipped with an HP-5 column (30 m × 0.32 mm, with 0.25 μm film) with injection at

290 °C and detection at 300 °C was used for the analysis. The temperature program was: at 100 °C (1.5 min), to 180 °C (6 °C/min), to 290 °C (4 °C/min), at 290 °C (13 min), to 300 °C (4 °C/min), and at 300 °C (20 min).

Analysis by HPLC

For the qualitative analysis of stump extractives with HPLC, 1 mg/mL dilutions of stump hot-water extracts were prepared in MeOH/H_2O (50/50, vol/vol) and filtrated through a 0.2-μm polytetrafluoroethylene (PTFE) filter. The HPLC analysis was performed with an Agilent 1290 LC (liquid chromatography) instrument equipped with a ZORBAX StableBond column (80 Å C18, 2.1 mm × 100 mm, 1.8 μm, 1200 bar), a ZORBAX SBC18 UHPLC guard column (2.1 mm, 1.8 μm), 1290 Infinity II Diode Array Detector, and a 6460 triple quadrupole mass spectrometer (LC/DAD/QQQ). The LC columns were maintained at 30 °C. Two solvents were used for the mobile phase: (A) 0.1% formic acid in UHQ water and (B) 0.1% formic acid in acetonitrile. The mobile phase flow rate was 0.4 mL/min. The run method was as follows: 5% B (from 0.0–20.0 min), 5–30% B (from 20.0–22.0 min), 30–80% B (from 22.0–24.0 min), and 80–5% B (from 24.0–25.0 min). Mass spectrometry analyses were performed in negative mode with a range of *m/z* 100–1200. The drying gas used was nitrogen at 10 L/min at 350 °C with a nebulizer pressure of 40 psi. The capillary voltage was 3100 V. The Bruker Data Analysis 3.2 software was used for data processing. Polydatin was used as an external standard for quantifying the hydrophilic compounds from the stump hot-water extracts.

Results and discussion

Due to an unfortunate random accident during sampling, four stump samples (weeks 4 and 25 from crushed stump, and zero-sample and 25-weeks sample from the stump heart) were lost, effectively making stump bottom the only whole stump series. Despite this, the authors of this article decided that presenting the remaining results from the other series as directive references would still be helpful.

Change in total dissolved solids

The combined TDS from *n*-hexane and water extracts in the studied stump samples are presented in Figure 2. Depending on assortment and storage time, TDS varied between 2.9% and 8.4% of dry weight. These are slightly higher concentrations to those reported by Hakkila, who found acetone extracts of *P. abies* stumps to total 2.2%–4.3% of dry matter.[11] Sjöholm has similarly reported central stump of *P. abies* to contain, on average, 2.8% and 1.5% of dry matter acetone and dichloromethane extractives.[12] It should be noted that acetone is more selective solvent than hot water and ASE-extraction (with more harsh extraction conditions) was utilized by us while Sjöholm used more mild Soxhlet-extraction.

In stump bottom, the hot-water extract totaled 70% of the extractives, 74% in crushed stump, and 73% in stump heart. In stump bottom, hydrophilic extractives ranged between 1.5% and 6.6% of dry matter, in crushed stump, 4.1%–5.8% of dry matter, and in stump heart, 1.9%–7.0% of dry matter. The total amount of *n*-hexane extractives ranged, in stump bottom, between 1.0% and 2.0% of dry matter, in crushed stump, 1.5% and 1.8% of dry matter, and in stump heart, 1.2% and 1.7% of dry matter. The crushed stump samples had consistently relatively high hydrophilic and lipophilic TDS, presumably caused by the bark material included in the samples. The root bark of Norway spruce is known for containing even up to 15%–29% of extractives.[13–15] In contrast, the lowest hydrophilic and lipophilic TDS seemed to be found in the debarked stump bottom samples. These results are comparable to those by Latva-Mäenpää, who found that *P. abies* stump wood contained 1.93% of dry matter acetone-soluble extractives.[16] Overall, there appeared to be no significant change in the total amount of lipophilic compounds in stump bottom or crushed stump samples during storage.

Of the studied lipophilic and hydrophilic compounds, about 40% and 50% were identifiable by GC-FID/MS, respectively. Unidentified compounds by GC-FID, in general, represent group of compounds that are poorly volatile and of higher molecular weight. Interestingly, unidentified hydrophilic compounds exhibited the most variation between individual stump samples. Variation between trees is not surprising by itself. The disparity in extractives content even between two trees of similar age can be high. Analogous results have also been reported regarding the extractives content between individual spruce logs.[17] Hydrophilic compounds in samples stored outside are generally lost more easily and prone to external influences, such as photodegradation via UV-light or leaching via rain.[18–21] In stump bottom, based on the identifiable (smaller molecular weight) compounds by GC, and despite the relatively low hydrophilic content of the zero-sample, an apparent

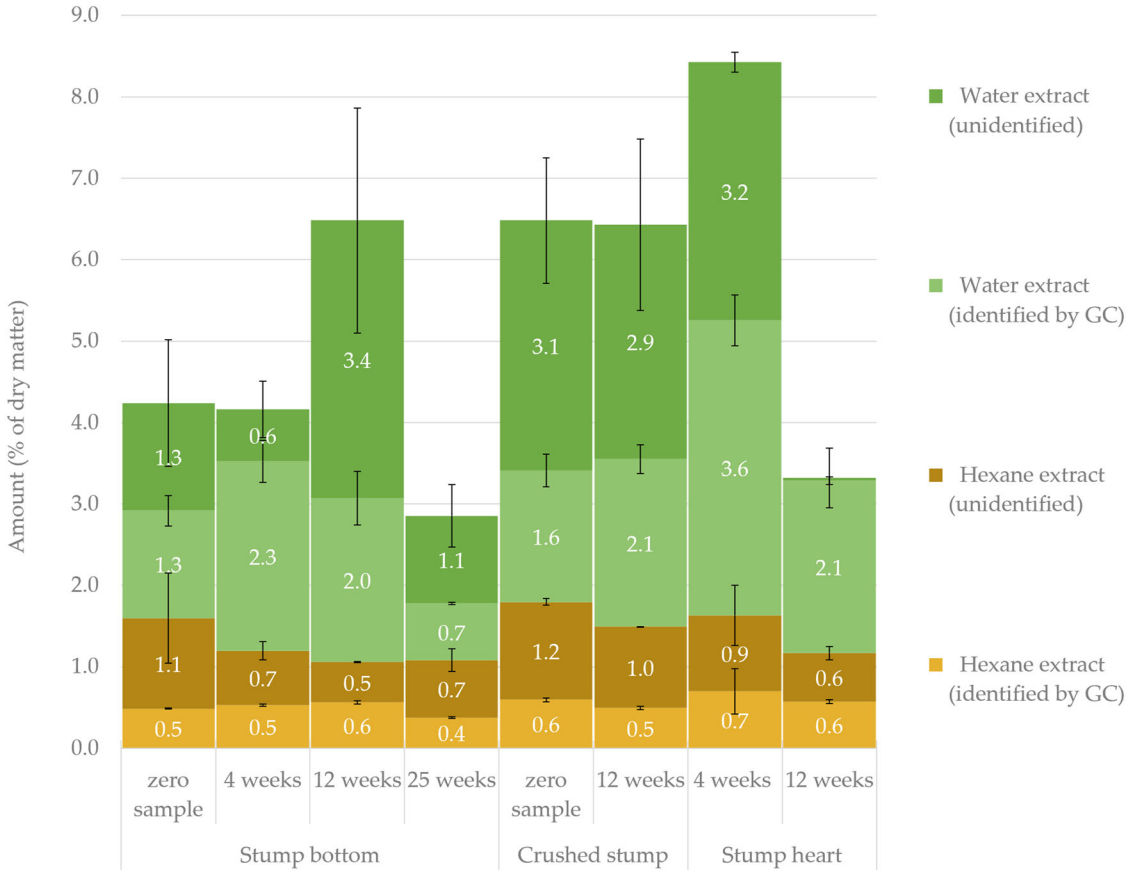

Figure 2. The total dissolved solids of the stump samples.

loss of hydrophilic extractives could be seen, aligning well with our expectations – namely, gradual loss of hydrophilic extractives. However, due to the relatively high variation between individual samples, especially in the amount of unidentifiable compounds, a definite conclusion on the decrease of stump extractives cannot be drawn based on these results.

Qualitative and quantitative results by GC-FID/GC-MS

The hydrophilic extractives groups of the stump samples are presented in Figure 3. Among the studied samples, approximately 43% of the extractives were identified, of which 79% were lignans, 8% sugars, 8% stilbene–glucosides and other aromatics, and 2% organic acids. Hydrophilic extractives, in general, seem to be most abundant in the crushed stump samples, explained by the inclusion of rootbark known for its high extractives content. *Picea abies* bark is also known to contain oligosaccharides and distilbenes unidentifiable with GC-FID.[17,22] This could also explain the abundance of unidentified hydrophilic compounds in the crushed stump samples. Lignan concentrations, on the other hand, appeared to be higher in the stump bottom and heart samples, in particular. This result would agree with the known data that lignan concentration in stumps is high, especially in the heart of the stump.[7,23] In stump bottom, despite the exceptionally high concentration of unidentified compounds in the stump bottom samples at week 12, the general trend appeared to be that the hydrophilic extractives content is decreasing during the storage. The 25-week samples from the stump bottom had, on average, 32% less hydrophilic compounds compared to the zero-sample. The high variation in unidentified compounds may reflect the fact that the excavator-cut stump pieces used for sampling were unevenly shielded in the storage pile from weathering. As has been previously demonstrated regarding bark in pile storage, it could be assumed that the stump samples in the middle of the pile have higher hydrophilic content than those in the outer layers. However, to confirm this, more studies should be conducted. For this study, assuming that all of the stumps in a given pile would be utilized for valorization, choosing between individual stump pieces regarding the location in a pile (whether in the middle or on the top) was not done. Only the stumps at the bottom of the pile were

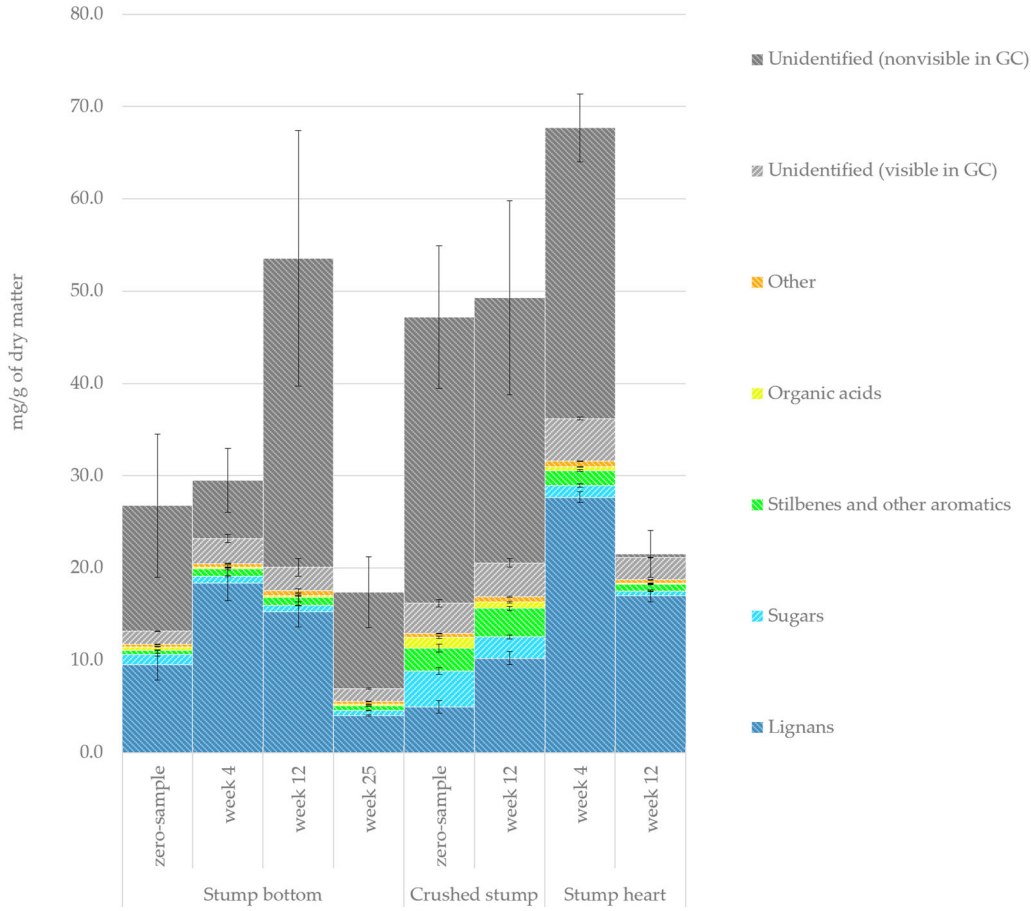

Figure 3. The hydrophilic extractives groups from the stump hot-water extracts as analyzed by GC-FID/MS.

disregarded due to a risk of contamination from the soil.

The concentration of lignans in the stump water extracts is presented in Figure 4. The greatest lignan concentration was found in spruce heart samples (on average 22 mg/g of dry matter) and the lowest in the crushed stump samples (on average 8 mg/g of dry matter). The average concentration of lignans in all the stump samples was 13.6 mg/g of dry matter. Much higher lignan concentrations have been found in the knotwood of spruce. Willför et al.[24] found as high as 6%–24% and Mansikkala et al.[25] 16% of lignans in *P. abies* knotwood by dry weight; 65%–85% of which was HMR. Latva-Mäenpää et al.[7] also found HMR lignan in some of the Norway spruce root neck samples at a concentration of ∼10% of total dry matter. Although several different variables could explain the difference, such as the soil of the growth area or the age of the trees, it appears that one of the significant reasons for the higher concentration of lignans reported by Latva-Mäenpää et al.[7] is due to their more precise way of cutting and separating the heartwood from the stump samples. Poller and Storkan[3] have demonstrated already in 1978 that the stump center is the most concentrated with extractives. Latva-Mäenpää has also demonstrated that the lowermost part of Norway spruce root neck has the highest saturation of lignans.[16] Thus, given that lignans are also localized predominately in and around the heartwood, it could be assumed that the more actual heartwood in the sample is included, the higher the lignan concentration would be.

Of the lignans discovered by us, in stump bottom, on average, 53% was HMR, 17% conidendric acid, 12% todolactol, 4% lignan A, 3% conidendrin, and 2% iso-HMR, isolariciresinol, lariciresinol, and other lignans, and 1% todolactol guiaiacyl ether, lignan A guaiacylglyceryl ether, nortrachelogenin, and oxo-matairesinol. In crushed stump, on average, 45% was HMR, 19% conidendric acid, 16% todolactol, 4% lignan A, 3% isolariciresinol and other lignans, 2% iso-HMR, lariciresinol, conidendrin, and 1% todolactol guiaiacyl ether, lignan A guaiacylglyceryl ether, nortrachelogenin, and oxo-matairesinol. In stump heart, on average, 56% was HMR, 15% conidendric acid, 10% todolactol, 4% conidendrin, 3% lignan A, 2% iso-HMR, lariciresinol, and 1% isolariciresinol, other lignans, todolactol guiaiacyl ether, lignan A

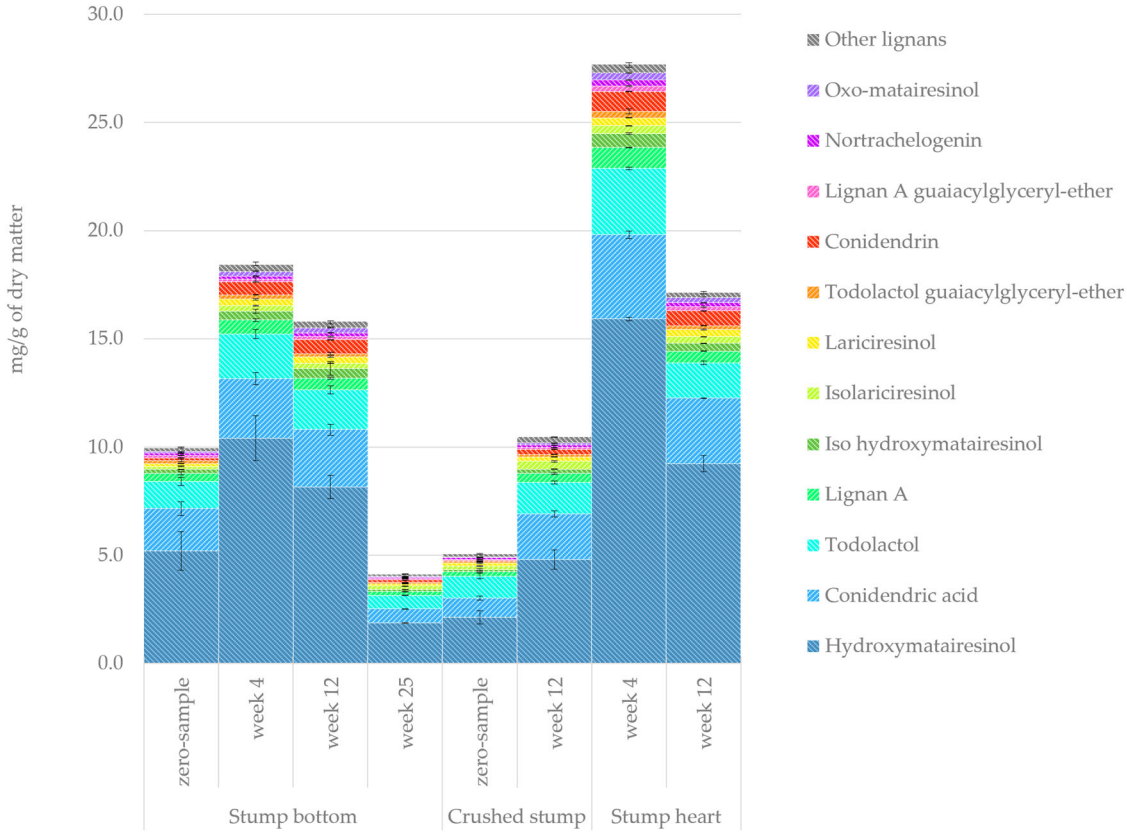

Figure 4. The lignans quantified by GC-FID/MS from stump hot-water extracts.

guaiacylglyceryl ether, nortrachelogenin, and oxo-matairesinol. It was also likely that among the unidentified hydrophilic compounds determined by GC-FID/MS, there was also a small amount of larger sesquilignans or dilignans, such as those discovered from *P. abies* knotwood by Mansikkala et al.[25] They found approximately 30% of the acetone extract of knotwood to consist of these larger lignan species.

Although the amount of lignans in the studied stump samples exhibited significant variation, indications of losses due to storage time could also be seen. Even despite the seemingly unusually low concentration at the stump bottom zero-sample, the apparent degradation of lignans after 25 weeks of storage was still 59%.

The amount of sugars in the stump hot-water extracts is presented in Figure 5. The average amount of sugars was approximately 1 mg/g of dry matter in the stump bottom and stump heart and 3 mg/g of dry matter in the crushed stump – a relatively insignificant amount compared to the amount of sugars in bark and sapwood. In *P. abies* bark, the hot-water extractable monosaccharides at 120 °C can reach up to 65 mg/g of dry matter.[17,26] The higher sugar concentration in the crushed stump can be explained by its carbohydrate-rich bark material. Of the identified sugars and their derivatives, in stump bottom, 37% was glucose, 23% galactose, 19% sucrose, 8% pinitol, 5% palatinose, and 2% inositol and other sugars, and 1% mannitol, arabitol. In crushed stump, 45% was glucose, 24% sucrose, 11% pinitol, 10% galactose, 2% inositol, mannitol and arabitol, and 1% palatinose and other sugars. In stump heart, 30% was glucose, 25% sucrose, 24% galactose, 8% pinitol, 4% palatinose, 3% other sugars, 2% arabitol, and 1% inositol and mannitol. The amount of sugars, mainly glucose and sucrose, appeared to decrease systematically during storage among all samples, while the relative proportion of galactose increased, following the pattern of degradation observed in previous studies regarding the degradation of *P. abies* bark extractives.[17,26] Interestingly, a relatively high amount of galactose and pinitol was also found in the stump samples. Pinitol is a well-researched methoxy derivative of chiro-inositol – a cyclitol with noted self-defensive and medicinal capabilities. For example, plants with high pinitol concentrations have traditionally been utilized to treat diabetes and cancer.[27]

The amount of stilbene–glucosides and other aromatic compounds from the stump samples is presented in Supplementary Figure S1. It should be noted that all of the stilbenoid species detected in the stump

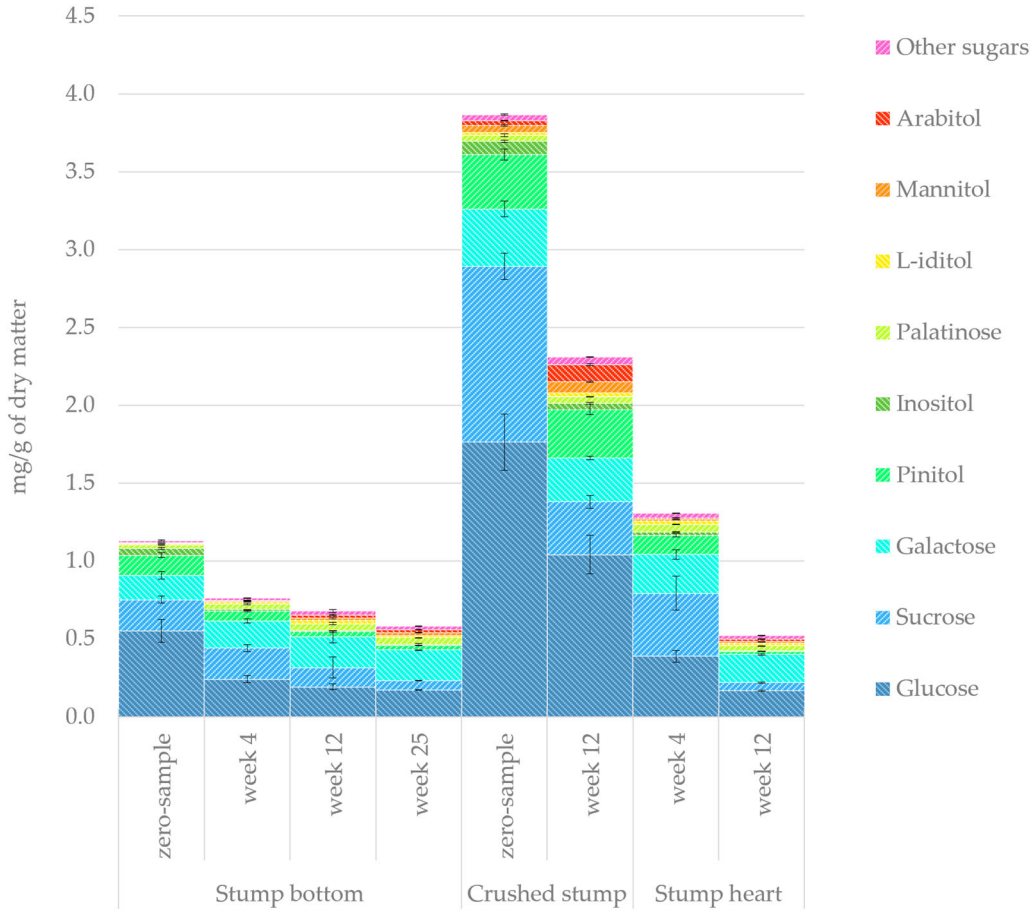

Figure 5. The sugars quantified by GC-FID/MS from stump hot-water extracts.

samples were stilbene–glucosides, namely isorhapontin, astringin, and piceid. Contrary to stump samples, aglycones of these stilbenoids have been detected in *P. abies* bark.[17,26] Among all samples, the average stilbene–glucoside concentration was 0.7 mg/g of dry matter. A slight elevation in stilbene–glucoside concentration was seen in the stump heart samples compared to the stump bottom. However, the highest concentration (2.0 mg/g of dry matter) was in the crushed stump samples. This was explained by the fact that stilbene–glucosides are concentrated primarily in the inner bark of *P. abies*, where their concentration can be as high as 24 mg/g of dry bark.[17] On the contrary, according to the results presented by Kebbi-Benkeder et al.,[28] the acetone extracts of knotwood and heartwood of *P. abies* do not contain stilbene–glucosides of any significant amount.

Of the stilbene–glucosides identified from the stump hydrophilic extracts, in stump bottom, 52% was piceid, 31% isorhapontin, and 17% astringin. In crushed stump, 75% was isorhapontin, 16% astringin, and 9% piceid. In stump heart, 41% was isorhapontin, 34% astringin, and 25% piceid. Other major (and quite resilient) aromatic compounds found in the stump samples were 1,3-(bisguaiacyl)-1,2-propandiol, 1-guaiacylglycerol, and coniferyl alcohol, with a combined concentration of 0.5 mg/g of dry matter in stump bottom, 0.6 mg/g of dry matter in crushed stump, and stump heart samples.

The amount of organic acids in the stump samples is presented in Supplementary Figure S2. The overall profile of the organic acids among the samples resembles very much the amount of sugars, as seen in Figure 5, where the crushed stump samples had the highest average concentration of 1.0 mg/g of dry matter and the stump bottom the lowest average concentration of 0.3 mg/g of dry matter – the decrease in extractives in all samples seemed to proceed in a very gradual fashion.

Of the identified organic acids, in stump bottom, 22% was citric acid, 14% L-aspartic acid, 13% gluconic acid, 11% malic acid, 9% quinic acid, 8% other acids, 7% vanillic acid, 5% shikimic acid, and 5% vanillic acid, and 2% threonic acid. In crushed stump, 31% was citric acid, 25% quinic acid, 9% malic acid, 7% gluconic acid and shikimic acid, 6% other acids, 4% L-aspartic acid, 3% vanillic acid, and 1% threonic acid. In stump heart, 21% was citric acid, 14% L-aspartic

acid and quinic acid, 12% gluconic acid, 9% malic acid, 8% vanillic acid, 7% other acids, 6% shikimic acid, and 1% threonic acid. Previous studies regarding *P. abies* bark extractives have demonstrated that the relative amount of gluconic acid, the most common organic acid in bark, is approximately equal to that of the combined amount of citric and quinic acids.[26] Citric acid has been utilized industrially, especially for its fermentation capabilities.[29] Compared to *P. abies* bark, the relative decrease in the prominence of gluconic acid in stump samples is most likely because the amount of gluconic acid depends on the amount of glucose in the samples, which is markedly lower in stumps. It has been demonstrated that gluconic acid may be obtained from glucose via enzymatic oxidation.[30] However, the formation of new organic acids due to microbial activity is very limited in the pile storage of stumps, unlike in the pile storage of bark, due to larger particle size and, thus, a lack of degradation caused by internal heating.[26,31,32] Among the different stump assortments, the relative increase in the amount of some important acids in the crushed stumps, such as the shikimate pathway acids, shikimic acid, and quinic acid (used in the synthesis of phenylpropanoid amino acids), is likely correlated to the inclusion of bark material in the crushed stump.

The lipophilic extractives groups of the stump samples are presented in Figure 6. Only approximately 32% of the lipophilic extractives were identified, of which in stump bottom, 54% was resin acids, 17% fatty acids, 11% diterpenoids, 10% lignans, 8% sterols, in crushed stump, 66% was resin acids, 14% fatty acids, 9% diterpenoids, 6% sterols, 4% lignans, and 1% other lipophilic compounds, and in stump heart, 54% was resin acids, 16% fatty acids, 12% diterpenoids, 10% lignans, 7% sterols, and 1% other lipophilic compounds. The lipophilic extractives remained relatively stable during 12 weeks of storage. The total amount of lipophilics divergence among the samples was only 13.0–16.5 mg/g of dry matter – a relatively small difference. However, in the stump bottom, unidentified compounds appeared to decrease by ~38% after 12 weeks of storage, while the overall drop in lipophilic extractives was 28.3%.

The slightly increased amount of fatty acids, diterpenoids, lignans, and sterols after 12 weeks of storage suggested that the observed decrease in unidentified compounds might be explained by hydrolysis reactions of triglycerides, steryl esters, oligomeric lignans, and diterpenoids. The hydrolysis of oligomeric lignans into simple lignans in alkaline and acidic conditions has been demonstrated with flax seed oligomeric lignans, namely secoisolariciresinol diglucosides.[33] As in the case of the hydrophilic extracts, the largest lignan concentration was found in the stump heart samples.

The amount of resin acids in the stump *n*-hexane extracts is presented in Figure 7. The average amount of resin acids in all samples was approximately 2.5 mg/g of dry matter, 4–5 times lower than the initial amount of resin acids in *P. abies* bark.[26] Of the identified resin acids, in stump bottom, 29% was dehydroabietic acid, 13% palustric acid, 10% levopimaric acid, isopimaric acid, and hydroxydehydroabietic acid, 7% abietic acid, 5% neoabietic acid and sandaracopimaric acid, 4% hydroxy resin acid, 3% pimaric acid, and 2% other resin acids. In crushed stump, 33% dehydroabietic acid, 14% hydroxydehydroabietic acid, 11% isopimaric acid, 10% hydroxy resin acid, 9% abietic acid, 5% palustric acid and levopimaric acid, 4% pimaric acid and sandaracopimaric acid, and 2% neoabietic acid and other resin acids. In stump heart, 29% was dehydroabietic acid, 12% palustric acid, 10% isopimaric acid and hydroxy dehydroabietic acid, 9% levopimaric acid, 8% abietic acid, 7% hydroxy resin acid, 5% neoabietic acid and sandaracopimaric acid, 3% pimaric acid, and 2% other resin acids. From zero-sample to 25 weeks of storage, the amount of resin acids in stump bottom decreased by 36%. In crushed stump, the apparent decrease was 21% after 12 weeks of storage. In stump heart, there was a similar 19% decrease in resin acids from week 4 to week 12 of storage. The total amount of dehydroabietic acid in samples remained relatively stable. In the stump bottom, dehydroabietic acid decreased only 14% in 25 weeks and hydroxydehydroabietic acid increased by 15%. A significant decrease was shown in palustric acid (74%) and levopimaric acid (76%), probably due to their having conjugated double bonds in their structure, which increase the reactivity (Diels–Alder reactions). The decrease in palustric acid and levopimaric acid has also been noted during bark storage.[26] In crushed stump, the most significant changes were the 73% decrease in neoabietic acid and 59% decrease in pimaric acid, while in stump heart 25% increase in palustric acid and 27% increase in levopimaric acid was observed and hydroxy resin acid decreased by 54%.

The amount of fatty acids in the stump samples is presented in Supplementary Figure S3. The total amount of fatty acids was 0.7 mg/g of dry matter in stump bottom and crushed stump and 0.9 mg/g of dry matter in stump heart. Prominent fatty acids in stump bottom were acid 18:2 (22%), acid 18:3 (17%), acid

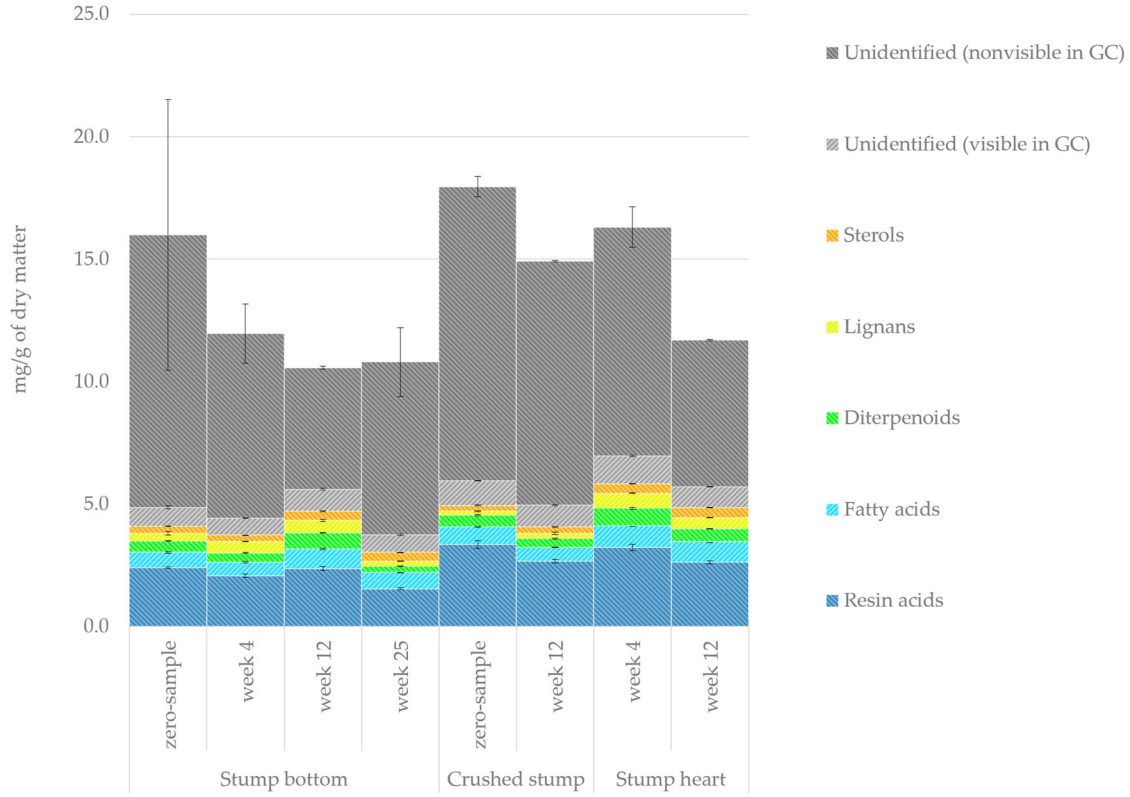

Figure 6. The lipophilic extractives groups from the stump *n*-hexane extracts as analyzed by GC-FID/MS.

18:1 (13%), acid 22:0 (9%), acid 16:0 (7%), acid 17:0 (6%), and acids 18:0 and 20:0 (5%). In crushed stump, acid 18:2 (19%), acid 18:1 (14%), acid 18:3 (13%), acid 22:0 (12%), acid 16:0 (7%), and acids 17:0 and 18:0 (5%). In stump heart, acid 18:2 (25%), acid 18:3 (17%), acid 18:1 (13%), acid 22:0 (9%), acid 16:0 (6%), acid 17:0, and acid 20:0 (5%). No significant change occurred during storage in the total amount of fatty acids. The maximum variation between the highest and the lowest fatty acid concentration was only 0.2 mg/g of dry matter. This result suggested that the 20% increase and the 23% decrease in the stump bottom and the crushed stump fatty acid content, respectively, at week 12 should be seen as part of the natural variation in stump lipophilic extractives rather than pure degradation related to storage.

The fatty acids are preserved much better in stump storage and sawlog bark[17] than in pile storage of bark.[26] This was probably due to the lack of thermal degradation associated with pile storage. However, it should be noted that there may have been degradation in the esterified fatty acids, which were not studied here (and thus were probably included among the unidentified lipophilic extractives group (see Figure 6).

The amount of diterpenoids in stump samples is shown in Supplementary Figure S4. The average amount of diterpenoids was 0.5 mg/g of dry matter in stump bottom, 0.4 mg/g of dry matter in crushed stump, and 0.6 mg/g of dry matter in stump heart. Of the identified diterpenoids, in stump bottom, 38% was thunbergol, 19% Δ^{13}-(*trans*)-neoabienol, 12% palustrol, 8% palustral, 5% pimarol, 4% pimaral and isopimarol and *cis*-abienol, and 3% epimanoyl oxide, 2% methyl neoabietate, and isopimaradiene, and 1% methyl abietatetraenoate. In crushed stump, 27% was thunbergol, 24% Δ^{13}-(*trans*)-neoabienol, 10% palustrol, 7% pimaral, 6% pimarol, 5% isopimarol and *cis*-abienol, 4% palustral, 3% methyl neoabietate and methyl abietatetraenoate, and 2% epimanoyl oxide and isopimaradiene. In stump heart, 39% was thunbergol, 14% Δ^{13}-(*trans*)-neoabienol, 12% palustrol, 7% palustral, 6% pimarol, 5% isopimarol, 4% pimaral and *cis*-abienol, 3% epimanoyl oxide, 2% methyl neoabietate and isopimaradiene, and 1% and methyl abietatetraenoate. Diterpenoids were more easily affected by storage than resin and fatty acids. In 25 weeks, diterpenoids appeared to decrease 43% in the stump bottom and 20% in the crushed stumps in 12 weeks. As in the case of fatty acids, the amount of diterpenoids in the stump bottom week 12 sample was notably higher than in previous weeks. Hence, it was interesting to note that looking at the total amount of lipophilic extractives (Figure 8), the amount of

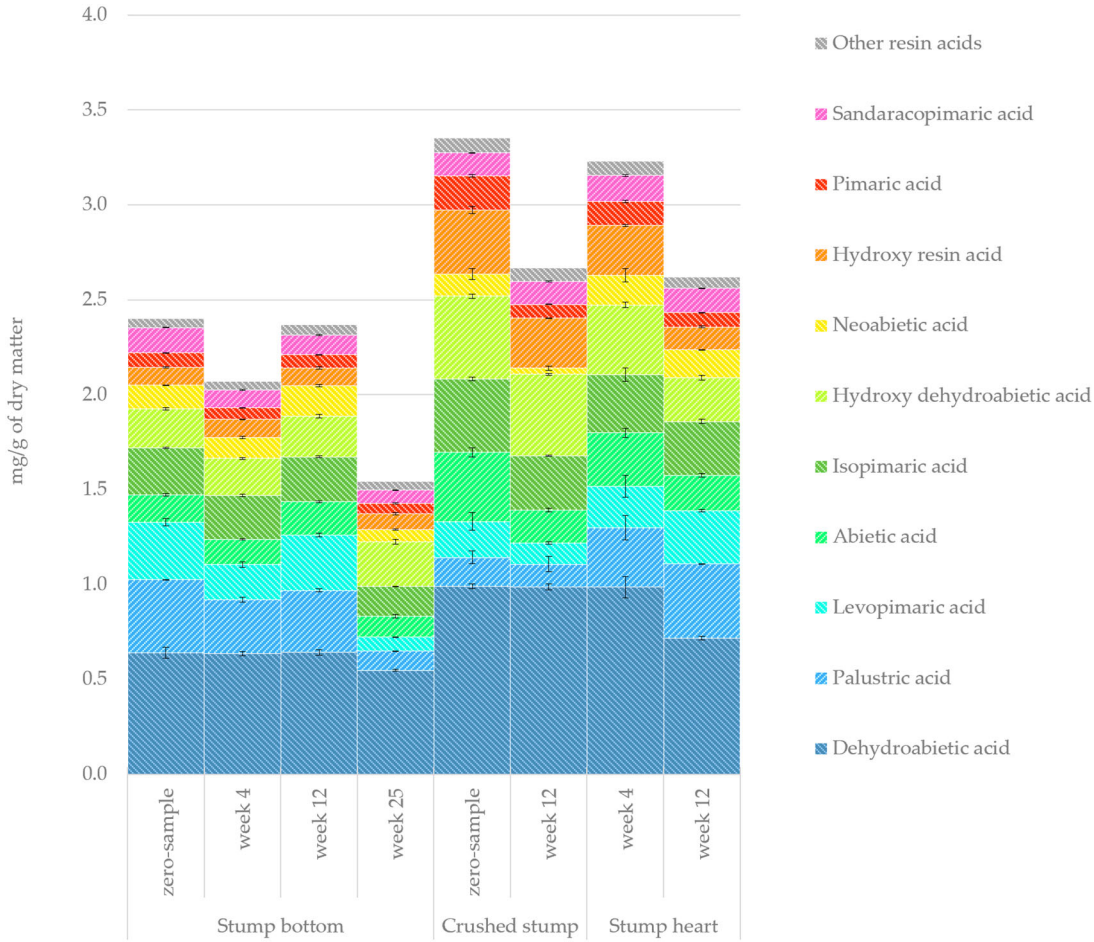

Figure 7. The resin acids quantified by GC-FID/MS from stump hexane extracts.

unidentified lipophilic compounds in the stump bottom at week 12 was also ~35% lower than what would be expected; the amount of unidentified compounds would be expected to follow a linear line of degression. Thus, the results indicated that the apparent increase in identified lipophilic compounds at the stump bottom week 12 was related to the simultaneous decrease of the unidentified compounds. An increase in fatty acids could be explained by increased hydrolysis of triglycerides among the unidentified compounds. Likewise, the observed increase in diterpenoids could be explained by the degradation of tri-, tetra-, or polyterpenoids among the unidentified compounds. The results suggested that the stump bottom samples at week 12 were particularly exposed to degrading conditions (direct sunlight or rainwater). Similar degradation divergence was observed in the bark samples of individual sawlogs.[17]

The total amount of lignans in the *n*-hexane extracts is presented in Supplementary Figure S5. It should be pointed out that, as also seen in Figures 3 and 4, lignans were by far the most prominent extractives group in the stump samples. While most (~97%) of the extracted and identified lignans were extracted *via* hot water, a small fraction (0.4 mg/g of dry matter on average) was also extractable *via* hexane. The *n*-hexane extracted lignan profile looked very similar to the hot-water extracted lignan profile in Figure 4, with HMR being the major lignan and constituting 48% of the *n*-hexane-extracted lignans in stump bottom, 52% in crushed stump and 51% in stump heart. The profiles of lariciresinol, conidendrin, and oxomatairesinol appeared especially similar, however the *n*-hexane extract contained roughly ten times less of them. Notably, some highly hydrophilic lignans were absent in the *n*-hexane extract, such as conidendric acid, todolactol, and lignan A, while other hydrophobic lignans not found in the water extracts, such as pinoresinol, were included. Interestingly, as the storage proceeds, the amount of pinoresinol appears to increase (at least proportionally to other lignans). This could be explained by monolignol radicals formed during the storage with a similar reaction as was demonstrated by Davin et al.[34] For the discussion about the degradation pattern of lignans, see above.

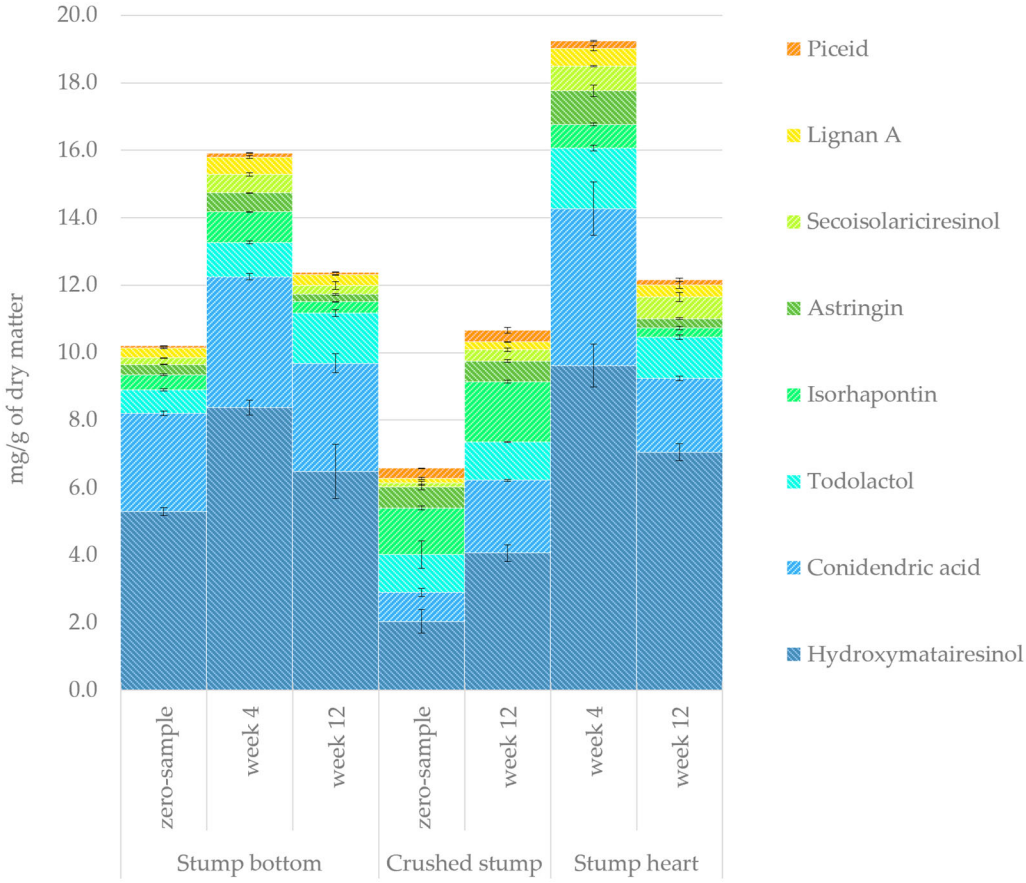

Figure 8. The hydrophilic compounds quantified by HPLC from stump hot-water extracts.

The quantified amount of sterols in the *n*-hexane extracts of the stump samples is presented in Supplementary Figure S6. The amount of sterols appeared to be relatively low (in general below 0.4 mg/g of dry matter). However, it should be noted that these sterols represented only the 'free sterols' in the *n*-hexane extract. The group of lipophilic compounds, which remained unidentified and invisible by the used GC-FID method, is presented in Figure 8. It could also be assumed that esterified sterols are included in that compound group. In the *n*-hexane extracts of *P. abies* bark, the amount of esterified sterols has been shown to be more significant than the amount of free sterols.[17,26] As in spruce stem bark, sitosterol and campesterol were the most prominent sterols in stumps. Of the identified sterols, in stump bottom, 52% was sitosterol, 21% campesterol, 11% 7-hydroxysitosterol, 7% sitostanol, 6% sitostadien-7-one, and 2% acid 24:0 monoglyceride and cholesteryl stearate. In crushed stump, 56% was sitosterol, 19% campesterol, 7% 7-hydroxysitosterol and sitostadien-7-one, 5% acid 24:0 monoglyceride, 4% sitostanol and 2% cholesteryl stearate. In stump heart, 56% was sitosterol, 19% campesterol, 9% 7-hydroxysitosterol, 6% sitostadien-7-one and sitostanol, 3% acid 24:0 monoglyceride and 2% cholesteryl stearate. Overall, the amount of sterols appeared to be very stable throughout the storage periods and stump parts. Non-esterified sterols derived from Norway spruce bark have also been shown to resist degradation, while the loss of esterified sterols is more significant.[26]

The results of the HPLC analysis of stump hot-water extracts are presented in Figure 8, in which the main identified phenolic compounds (lignans and stilbene–glucosides) are presented. Except for secoisolariciresinol, all these compounds were also found and quantified by GC-FID (Figure 4 and Supplementary Figure S2). By comparison, the GC-FID method yielded an average 10.5% higher concentration for the extractives than the HPLC method. However, the average divergence in the concentration was markedly dependent on the stump sample. In the stump bottom samples, the GC-FID results were only an average of 1.0% higher than those from HPLC. Similarly, the GC-FID results were only 1.7% higher in the crushed stump samples. However, in the case of the stump heart samples, the GC-FID results were 26.9% higher than those presented in Figure 8 and focused heavily on the quantified amount of HMR in the 4-weeks stump storage sample.

The differences between the GC and HPLC results could generally be attributed to the differences in the analysis method itself, sample preparation, and the used standards. The HPLC samples were filtered prior to analysis, while the GC-FID samples were not. The HPLC samples were also quantified against the polydatin standard, while the GC samples were quantified by the internal standards, heneicosanoic acid and betulin. However, the unusually significant difference in the HMR concentration in the stump heart sample at week 4 between GC-FID and HPLC appears to be more related to the nature of the sample matrix. The HPLC samples were analyzed from different extracts than those used for the GC analyses. However, the same ground stump heart material was used in both cases. This indicated that even after grinding, the used stump powder remains inhomogeneous with respect to lignan concentration and should have undergone more thorough mixing. Concerning lignan concentration, the inhomogeneity was highly localized toward the pith of the stump; thus, the acquired stump heart material would also contain material with varying lignan concentrations.

The stilbene–glucoside concentrations (isorhapontin, astringin, and piceid) of the HPLC samples were, on average, 3.8 mg/g of dry matter greater than in GC-FID samples. Similar observations have also been made in a previous study regarding GC-FID and HPLC analyzed Norway spruce bark.[17] The four most prominent lignans identified by GC-FID, namely, hydroxymatairesinol, conidendric acid, todolactol, and lignan A were also identified by the HPLC method. It should be noted that the compounds that could not be reliably identified were left out of Figure 8.

The gained chromatographic results confirm that HMR is the most prominent lignan in Norway spruce. Commercial HMR products, such as HMRlignan™ made from Norway spruce knotwood by a Swiss company Linnea, have been available as dietary supplement capsules since 2006.[2] In addition, US company Swanson Health Products also sells HMR extracted from spruce knotwood in 60 mg × 40 mg HMR capsules.[35] According to the GC-FID results of the crushed stump, the average amount of HMR was 3.5 mg/g of dry matter, in stump bottom 6.4 mg/g of dry matter, and in stump heart 12.6 mg/g of dry matter. The pattern of these results agrees with the idea that lignans are mainly concentrated in the heartwood near the pith of the stump.

To conservatively estimate the potential of producing HMR capsules (as described earlier) from *P. abies* stumps, it can be concluded that if the dry mass of a single spruce stump is assumed to be 30 kg and the extractable HMR concentration of crushed bark 3 mg/g of dry matter, a single crushed stump will yield 90 g of pure HMR lignan, which would translate to 37.5 packages of 60 mg × 40 mg HMR capsules. If one package were sold for 10 USD, this would place a 12.5 USD/kg value for the stumps, only based on the HMR concentration.

Conclusions

Based on this study, it can be concluded that *P. abies* stump material presents an exciting option for the isolation and further purification of extractives, especially lignans. Higher intensity in lignan concentration seems to be located, especially at the bottom and heart of the stump material; however, the selective isolation of lignan-rich parts of the stump might prove to be impractical for upscaling. Precut and piled stumps exhibit significant variation in their extractives content. Lipophilic stump extractives are minor components and quite stable in all samples, even at the stump bottom stored for 25 weeks. Hydrophilic extractives are predominant; however, storage appears to decrease their amount in stump bottom and stump heart. However, because of the high extractives variation between samples, the perceived effects of storage may be coincidental.

It seems, nevertheless, reasonable that if stumps are stored for extractives utilization, they should be stored as intact as possible because any cutting of the stump material would dramatically increase the likelihood of oxidation, UV-light, and potential microbial attack on the stump extractives. While inconclusive, the results from the crushed whole stump, retaining much of its initial value in all samples in terms of total extractives content, certainly support this assumption (Figures 2–4). However, further studies would need to be conducted to confirm this as well as the possibility and effect of shielding stump material from UV-light and weathering during storage. In addition, the viability of collecting the hydrophilic extractives easily lost due to leaching from the storage piles is also worth future investigation.

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